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Iron regulatory protein prevents binding of the 43S translation pre-initiation complex to ferritin and eALAS mRNAs

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Translation of ferritin and erythroid 5-aminolevulinate synthase (eALAS) mRNAs is regulated by iron via mRNA–protein interactions between iron-responsive elements (IREs) and iron regulatory protein (IRP). In iron-depleted cells, IRP binds to single IREs located in the 5′ untranslated regions of ferritin and eALAS mRNAs and represses translation initiation. The molecular mechanism underlying this translational repression was investigated using reconstituted, IRE–IRP-regulated, cell-free translation systems. The IRE–IRP interaction is shown to prevent the association of the 43S translation pre-initiation complex (including the small ribosomal subunit) with the mRNA. Studies with the spliceosomal protein U1A and mRNAs which harbour specific binding sites for this protein in place of an IRE furthermore reveal that the 5′ termini of mRNAs are generally sensitive to repressor protein-mediated inhibition of 43S pre-initiation complex binding.

Key words: eALAS/iron-responsive elements/post-transcriptional regulation/RNA–protein interactions/ribosome binding

Introduction

Translation of most cellular mRNAs requires a m^7GpppX cap structure at the 5′ terminus of the mRNA for efficient binding of the 43S pre-initiation complex. Some viral mRNAs, particularly those belonging to the picornavirus family, as well as a few cellular mRNAs, initiate translation internally within the 5′ untranslated region (UTR) in a cap-independent way (Sonenberg, 1991; Oh and Sarnow, 1993; Jackson *et al.*, 1994). The pathways for both cap-dependent and -independent translation initiation are still incompletely defined. Cap-dependent initiation involves a number of biochemically well-characterized initiation factors (eIFs), including proteins with affinity for the cap structure and RNA helicases, which are thought to assist entry of the 43S pre-initiation complex (40S small ribosomal subunit plus the ternary complex containing the initiator $tRNA_i^{Met}$, eIF-2 and GTP) to the mRNA. In the case of internal initiation, the 43S complex binds to the mRNA with the assistance of complex structural motifs within the mRNA and auxiliary cellular factors (Jackson *et al.*, 1994). Unless it binds directly to the AUG translation initiation codon (Kaminski *et al.*, 1990), the 43S complex searches along the 5′ UTR until it reaches the AUG initiator codon (Kozak, 1989). At this point, GTP is hydrolysed, initiation factors are released and the 60S large ribosomal subunit joins to form an 80S translation-competent ribosome (Rhoads, 1988; Sonenberg, 1988; Merrick, 1992).

Many genes whose products are involved in diverse cellular processes, such as basic metabolism, developmental programmes or growth and differentiation, are known to be translationally regulated (summarized in Gray and Hentze, 1994). An intensively studied example of translational regulation by a repressor protein is the synthesis of ferritin. The expression of this intracellular iron storage protein was found to be regulated by iron almost 50 years ago (Granick, 1946) and has been known as an example of translational regulation for about 20 years (Zähringer *et al.*, 1976). The same protein that binds to and represses ferritin mRNA translation also regulates the translation of erythroid 5-aminolevulinate synthase (eALAS) mRNA, an enzyme involved in haem synthesis (Cox *et al.*, 1991; Dandekar *et al.*, 1991; Melefors *et al.*, 1993). Iron regulatory protein (IRP, formerly called IRF, IRE-BP, FRP or P-90) represses ferritin and eALAS mRNA translation by binding to a 5′ UTR motif, the iron responsive element (IRE). This stem–loop structure (approximately −5.0 kcal/mol) is not sufficiently stable to impede translation by itself, but in conjunction with IRP suffices to mediate translational control both *in vivo* and *in vitro* (Aziz and Munro, 1987; Hentze *et al.*, 1987a; Walden *et al.*, 1989; Gray *et al.*, 1993). The activity of IRP, which is an iron–sulfur cluster protein, is regulated by changes in its cluster in such a way that it binds to IREs with high affinity in iron-starved cells (Constable *et al.*, 1992; Haile *et al.*, 1992a,b; Emery-Goodman *et al.*, 1993). In addition to iron deprivation, nitric oxide release stimulates IRE binding by IRP (Drapier *et al.*, 1993; Weiss *et al.*, 1993; Pantopoulos *et al.*, 1994).

While much has been learnt about IRP, the mRNAs to which it binds and the signal transduction to it, surprisingly little is known about the mechanism of how IRE–IRP complexes affect translation. Ferritin and eALAS mRNAs do not contain upstream open reading frames. Changes in the length of their poly(A) tails following iron regulation have not been observed and IRP-mediated translational repression *in vitro* does not require a polyadenylated mRNA (Swenson *et al.*, 1991; Gray *et al.*, 1993). The conserved position of the IRE within the cap-proximal 40 nucleotides of ferritin and eALAS mRNAs from different species is functionally important, because an IRE only poorly mediates translational regulation when moved further than 60 nucleotides downstream from the cap structure (Goossen *et al.*, 1990; Goossen and Hentze, 1992). Since IRE-like translational repression can be achieved by substitution of the IRE with binding sites for

proteins which play no physiological role in eukaryotic translation (such as the spliceosomal protein U1A and the bacteriophage MS2 coat protein) and are therefore unlikely to repress translation by specific interactions with translation initiation factors, it was suggested that these RNA–protein complexes and perhaps the IRE–IRP complex block translation sterically (Stripecke and Hentze, 1992; Stripecke *et al.*, 1994).

In this report we have investigated how IRP and the U1A protein, as an example of a steric inhibitor of translation initiation, affect ribosome access to the mRNA. We have employed a biochemical approach taking advantage of cell-free systems reconstituted to study repressor protein-mediated translational control *in vitro* (Stripecke and Hentze, 1992; Gray *et al.*, 1993). We demonstrate that binding of both repressor proteins inhibits the association of the 43S translation pre-initiation complex with the mRNA.

Results

IRP binding causes redistribution of ribosome-engaged mRNAs into complexes smaller than 80S

To investigate which components of the translational machinery associate with an IRE-regulated mRNA in the presence of IRP, translation initiation assays were performed using ^{32}P-labelled mRNAs and translation-competent extracts from wheat germ or rabbit reticulocytes. The resulting initiation complexes were resolved by centrifugation through linear sucrose gradients. Following fractionation, the distribution of mRNA was assessed by RNA extraction and analysis on denaturing polyacrylamide gels or by counting of the radioactivity associated with each fraction. Comparative experiments established that scintillation counting quantitatively reflected non-degraded full-length mRNAs (data not shown). The assays were routinely performed in the presence of cycloheximide, an antibiotic which interferes with the peptidyl transfer reaction (Obrig *et al.*, 1971). Thus, 80S ribosomes which assemble on the mRNA at the AUG initiator codon are trapped and run-off is prevented.

Initially, three different mRNAs were compared in these assays. IRE-wt bears an intact ferritin IRE upstream of a chloramphenicol acetyltransferase (CAT) reporter open reading frame (ORF) in a position equivalent to that of the IRE in ferritin mRNA (Figure 1) and is repressed by IRP *in vitro* (Gray *et al.*, 1993). IRE-mut is identical to IRE-wt except for the deletion of a single nucleotide from the IRE, which renders it incapable of high affinity IRP binding and translational repression, but is unlikely to alter the structure of the mRNA as a whole (Gray *et al.*, 1993). A transcript encoding the U1A protein which contains no ferritin-, eALAS- or IRE-related sequences was used as an additional control (Scherly *et al.*, 1989). All mRNAs were generated by *in vitro* transcription using the m^{7}GpppG cap analogue under conditions optimized to ensure that >95% of transcripts were capped.

Being a plant-derived translation system, wheat germ extract is devoid of endogenous IRP (Walden *et al.*, 1989) and thus allows control of IRP activity experimentally by addition of recombinant protein (Gray *et al.*, 1993). When initiation assays were performed with the three different

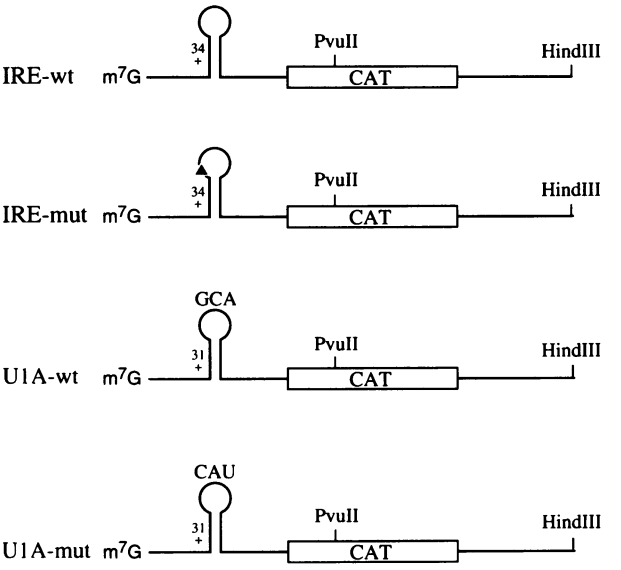

Fig. 1. Diagram of the CAT reporter mRNAs used in this study. The open reading frame encoding chloramphenicol acetyltransferase is denoted by an open box. The position of the IRE in the 5′ UTR is quoted with reference to the distance of the conserved unpaired C residue in the IRE (Goossen and Hentze, 1992; not indicated here) from the cap structure. The black triangle symbolizes an IRE which lacks the first nucleotide of the conserved loop. The position of the U1A binding site is quoted with reference to the distance of the insertion site from the cap structure (Stripecke and Hentze, 1992). The RNA sequences indicated above the loop of U1A-wt and U1A-mut represent the only differences between the two transcripts. The AUG initiator codon is located at +103 and +102 in IRE-wt and IRE-mut respectively and at +111 in U1A-wt and U1A-mut. The restriction sites used to linearize the plasmids prior to transcription are indicated.

mRNAs, their resulting sedimentation patterns through linear gradients of 10–30% sucrose were roughly similar (Figure 2, dotted squares). The mRNA concentration was limited to maximize the proportion of mRNA which was ribosome-associated and sedimented in fractions 1–13 (data not shown). Even in the presence of 0.5 mM cycloheximide, the majority of ribosome-associated mRNAs sedimented faster than 80S, the expected size of monosomes (a significant proportion of mRNAs assayed in rabbit reticulocyte lysate also sedimented faster than 80S, see below). The same pattern emerged with anisomycin, another elongation inhibitor, and [^{35}S]Met incorporation was found to be completely inhibited by as little as 0.1 mM cycloheximide or anisomycin (data not shown), indicating that incomplete inhibition by cycloheximide is unlikely to account for the faster sedimentation rate. These particles more likely represent 80S monosomes with additional 43S pre-initiation complexes associated with the 5′ UTR, which are known to form when mRNAs have a sufficiently long 5′ UTR (Kozak, 1991). Mild digestion of translation complexes with RNase A prior to centrifugation did not allow us to distinguish unambiguously between these possibilities. Addition of recombinant IRP to the translation initiation assays had little effect on the sedimentation pattern of U1A and IRE-mut mRNAs (Figure 2A and C, black diamonds), whereas IRE-wt mRNA was largely displaced from fractions 1–13 and appeared to co-sediment with the small ribosomal subunit in fractions 17–21 (Figure 2B, black diamonds). This

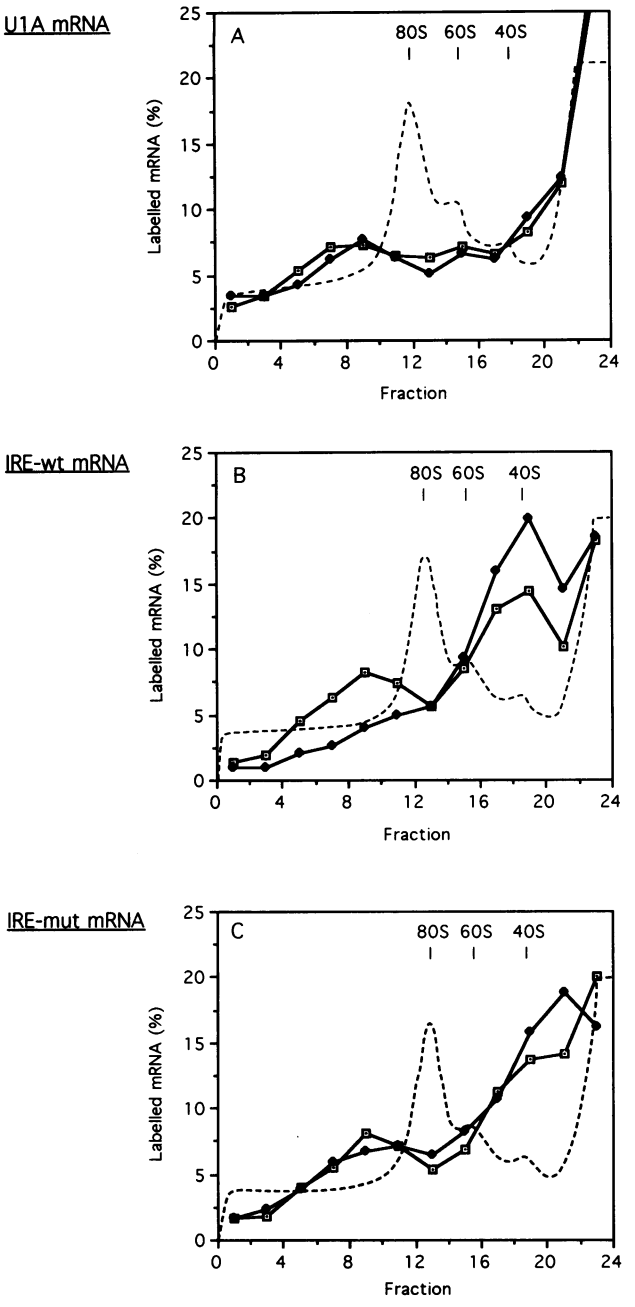

Fig. 2. Sucrose gradient profiles of translation initiation assays performed in wheat germ extract. Full-length mRNA transcripts of (**A**) U1A, (**B**) IRE-wt and (**C**) IRE-mut were assayed in the absence or presence of IRP. Initiation complexes were analysed on 10–30% linear sucrose gradients and fractionated. The labelled mRNA in the fractions is expressed as a percentage of total counts recovered. The small effect of IRP on IRE-mut mRNA (C) is not representative of findings in similar experiments. The percentage of mRNA is plotted against the fraction number (dotted squares, −IRP; filled diamonds, +IRP). The dashed line denotes the A_{254} absorption profile, which was identical for gradients −/+IRP. The positions of 40S, 60S and 80S ribosomal particles are indicated.

result suggested that the translationally repressed IRE-wt mRNA was associated with a stalled pre-initiation complex.

To examine further this apparent association of repressed IRE-wt mRNA with 43S pre-initiation complexes, inhibitors of translation which induce accumulation of defined initiation intermediates were included in the assays. The m^7GpppG cap analogue sequesters initiation factors with affinity for the cap structure of the mRNA. Sequestration of these factors, which facilitate binding of the 43S complex to the mRNA, leads to the accumulation of mRNA in messenger ribonucleoprotein particles (mRNPs) in the top fractions of the gradient (Anthony and Merrick, 1992). The non-hydrolysable GTP analogue GMP-PNP induces the accumulation of 43S pre-initiation complexes stalled at the initiation codon, because GTP hydrolysis is required prior to the joining of the 60S ribosomal subunit (Hershey and Monro, 1966; Anthony and Merrick, 1992). Hence GMP-PNP and m^7GpppG can be used to localize mRNAs engaged with 43S pre-initiation complexes (known as 48S complexes) or no ribosomal subunits respectively. The sucrose concentration of the gradients was adjusted to 5–25% to improve the resolution between complexes which sediment more slowly than 80S. Paradoxically, IRP-bound IRE-wt mRNA displayed similar sedimentation rates (~40S) in the presence of m^7GpppG or GMP-PNP or in the absence of analogues (data not shown). The sedimentation rate of IRP-repressed mRNA at around 40S should therefore not be taken as evidence for the association of IRP-repressed mRNAs with the small ribosomal subunit.

High resolution analysis of mRNA particles inhibited by IRP

We reasoned that the sedimentation of IRE-wt mRNA could have been influenced by proteins in the extract which non-specifically bound to the 860 nucleotide long mRNA, a situation that has also been encountered by other investigators (e.g. Kozak, 1980; Sonenberg *et al.*, 1981). Since the elements for IRE−IRP regulation are confined to the 5′ UTR, truncated mRNAs (218 nucleotides in length) were transcribed from templates linearized at the *Pvu*II site 115 nucleotides into the CAT ORF (Figure 1). These shortened mRNAs should form initiation complexes and may allow the resolution of bona fide 48S pre-initiation complexes from mRNPs. As expected, the shortened IRE-wt mRNAs were efficiently bound by ribosomes in the absence of IRP (Figure 3A, dotted squares). In contrast to their longer counterparts, they were predominantly found in the top fractions of the gradient when incubated with the m^7GpppG cap analogue (Figure 3B) and distinctly heavier complexes were observed when GMP-PNP was included (Figure 3C, dotted squares), marking the position of 48S complexes. The IRP repressor protein reduced the fraction of ribosome-bound mRNA to background levels and appeared to redistribute it into the top fractions of the gradient (Figure 3A, black diamonds). When the m^7GpppG cap analogue was included, the mRNA sedimented similarly in both the presence and absence of IRP, indicating that IRP itself did not affect the sedimentation rate of the mRNA (Figure 3B). While in the absence of IRP a significant fraction of the mRNA had sedimented as 48S complexes with the inclusion of GMP-PNP, IRP caused displacement of this mRNA into the top fractions of the gradient (Figure 3C). These results strongly suggest that IRP excludes the mRNA from engagement with 43S pre-initiation complexes. When IRE-mut mRNA was analysed

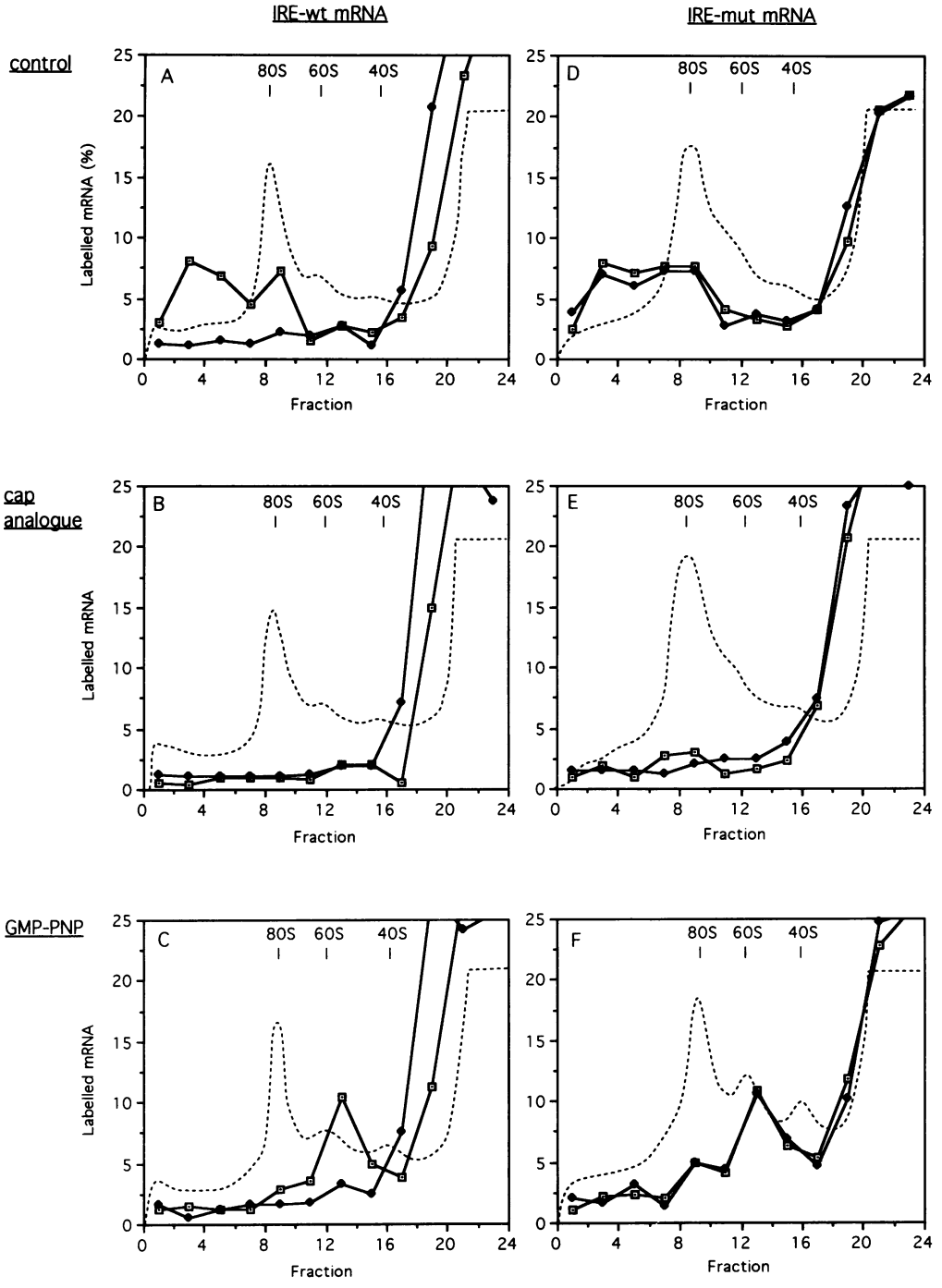

Fig. 3. Sucrose gradient profiles of translation initiation assays performed in wheat germ extract with shortened mRNAs. IRE-wt (**A**–**C**) and IRE-mut (**D**–**F**) mRNAs were assayed (dotted squares, −IRP; filled diamonds, +IRP) and analysed on 5–25% linear sucrose gradients. The assays contained either no analogue (A and D), the m^7GpppG cap analogue (B and E) or GMP-PNP (C and F). The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A_{254} absorption profile, which was identical for gradients −/+IRP. The positions of 40S, 60S and 80S ribosomal particles are indicated.

in the same manner (Figure 3D–F), this mRNA was unaffected by IRP under all conditions, demonstrating the specificity of the above results.

In rabbit reticulocytes, ferritin and eALAS mRNA translation is regulated by endogenous IRP (Walden *et al.*, 1988); IRE-wt mRNA is thus partially repressed in translation extracts from these cells (Figure 4, lane 3). To augment the translational repression of IRE-wt mRNA, the endogenous IRP was supplemented with recombinant human IRP (Figure 4, lane 4); conversely, translation was de-repressed when the endogenous IRP was competitively sequestered with IRE-containing oligoribonucleotides (Figure 4, lane 2). Employing this approach, the mechanism of translational repression by IRP was investigated in this mammalian translation system. As shown in Figure 5, sucrose gradient analysis of short IRE-wt and IRE-mut mRNAs in the presence and absence of IRP yielded similar results to the wheat germ system. The effect of

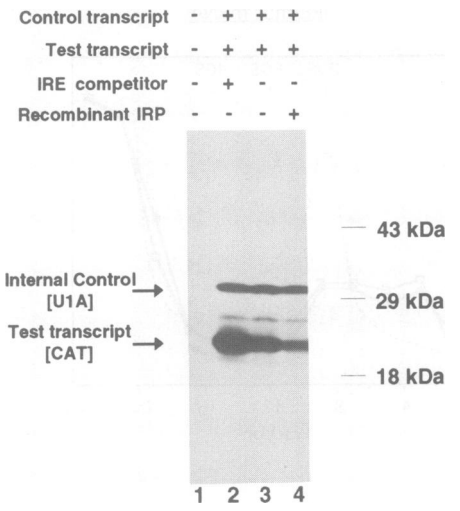

Fig. 4. Translation of IRE-wt mRNA under repressing and de-repressing conditions in rabbit reticulocyte lysate. U1A (1 ng) and 2.5 ng full-length IRE-wt mRNAs were co-translated in the presence of either 15 ng IRE competitor RNA (lane 2) or 125 ng recombinant human IRP (lane 4). ^{35}S-labelled translation products were analysed by SDS–PAGE and fluorography. Molecular weight markers are indicated on the right and the positions of the U1A and CAT proteins are shown on the left.

IRP on IRE-wt mRNA was even more pronounced, strengthening the argument that IRP affects 43S pre-initiation complex binding.

IRP blocks 43S translation pre-initiation complex binding to the mRNA.

The sedimentation of IRE-wt mRNA in the presence of IRP suggests that the small ribosomal subunit is not bound to it. The question then arises as to whether this reflects the mechanism by which IRP represses translation or whether it could be explained by dissociation of the 43S complex during the analytical procedure. Since GMP-PNP stalls the 43S pre-initiation complex at the AUG initiator codon prior to joining of the large ribosomal subunit, interactions between the initiator tRNA and the AUG codon might stabilize a 48S complex that would otherwise dissociate. Assuming that IRP did not prevent the binding of the 43S complex but its migration to the AUG codon, this could result in a 43S complex which is associated less strongly with the mRNA and which may therefore be lost by dissociation during the experimental procedure. To address this concern directly, translation assays were performed with edeine, an antibiotic which interferes with AUG recognition by the 43S complex and thus prevents possible AUG/initiator tRNA-mediated stabilization of 48S complexes (Kozak and Shatkin, 1978; Anthony and Merrick, 1992).

When rabbit reticulocyte IRP was sequestered by competitor IREs, the shortened IRE-wt mRNA sedimented in 48S complexes when assayed in the presence of either GMP-PNP (Figure 6B, dotted squares) or edeine (Figure 6C, dotted squares). Complexes sedimenting faster than 48S were also observed (see also Figure 5C and E); these are indicative of multiple 43S pre-initiation complexes associated with the mRNA (see above). In both cases, recombinant IRP caused the loss of 43S complexes from the mRNA (Figure 6B and C, black diamonds). IRP had no effect on the sedimentation of short IRE-mut mRNA incubated with GMP-PNP or edeine (data not shown). Furthermore, similar initiation assays with IRE-wt and IRE-mut mRNAs in wheat germ extract confirmed the results described above (data not shown). Thus, IRP prevents the binding of the 43S pre-initiation complex to the mRNA rather than inhibiting its progression to the initiation codon.

Steric inhibition of translation by the U1A protein mechanistically resembles ferritin regulation by IRP

Next, we probed the mechanism by which steric repressors that bind to cap-proximal RNA sites affect translation. Two examples of steric regulators have been described: the spliceosomal U1A protein and the bacteriophage MS2 coat protein (Stripecke and Hentze, 1992; Stripecke *et al.*, 1994). CAT reporter mRNAs, one with a high affinity wild-type (U1A-wt) and one with a mutant binding site (U1A-mut) which is not repressed in the presence of U1A protein (Stripecke and Hentze, 1992 and data not shown), were used to examine how the U1A protein inhibits translation initiation. The binding sites were located in a position similar to that of the IRE in IRE-wt mRNA (Figure 1).

In the absence of U1A protein, both short U1A-wt and U1A-mut mRNAs bound to ribosomes when assayed in rabbit reticulocyte lysate (Figure 7A and B, dotted squares). The U1A protein differentially removed U1A-wt, but not U1A-mut mRNA, from these lower fractions of the gradient (Figure 7A and B, black diamonds). The quantitative differences between IRP (Figure 5) and U1A (Figure 7) as repressors of 80S ribosome formation are in good accord with previous *in vitro* translation studies (Stripecke and Hentze, 1992; Gray *et al.*, 1993). In analogy to the approach used for IRP, definition of whether repressed U1A-wt mRNA was associated with 43S complexes was achieved using m^{7}GpppG and GMP-PNP. Similar to IRP, the UIA protein specifically blocked translation initiation by preventing access of the 43S ribosomal subunit to the mRNA (Figure 7C and E).

Discussion

Regulation of ferritin and eALAS mRNAs

The mechanism of translational repression by IRP has been investigated biochemically using *in vitro* initiation assays, an indicator mRNA that bears a human ferritin H-chain IRE in a position identical to that of ferritin IREs and highly purified recombinant human IRP. Collectively, the data demonstrate that IRP prevents binding of the 43S translation pre-initiation complex to the mRNA. The previous evaluation of the CAT reporter mRNAs, which showed that an intact ferritin or eALAS IRE was necessary and sufficient to mediate translational control *in vivo* (Hentze *et al.*, 1987b; Melefors *et al.*, 1993), and in the cell-free translation systems utilized here (Gray *et al.*, 1993; Melefors *et al.*, 1993) allow the conclusion that the results reported here reflect the cellular mechanism by which IRP represses the translation of ferritin, eALAS and possibly other mRNAs in iron-starved cells. Results

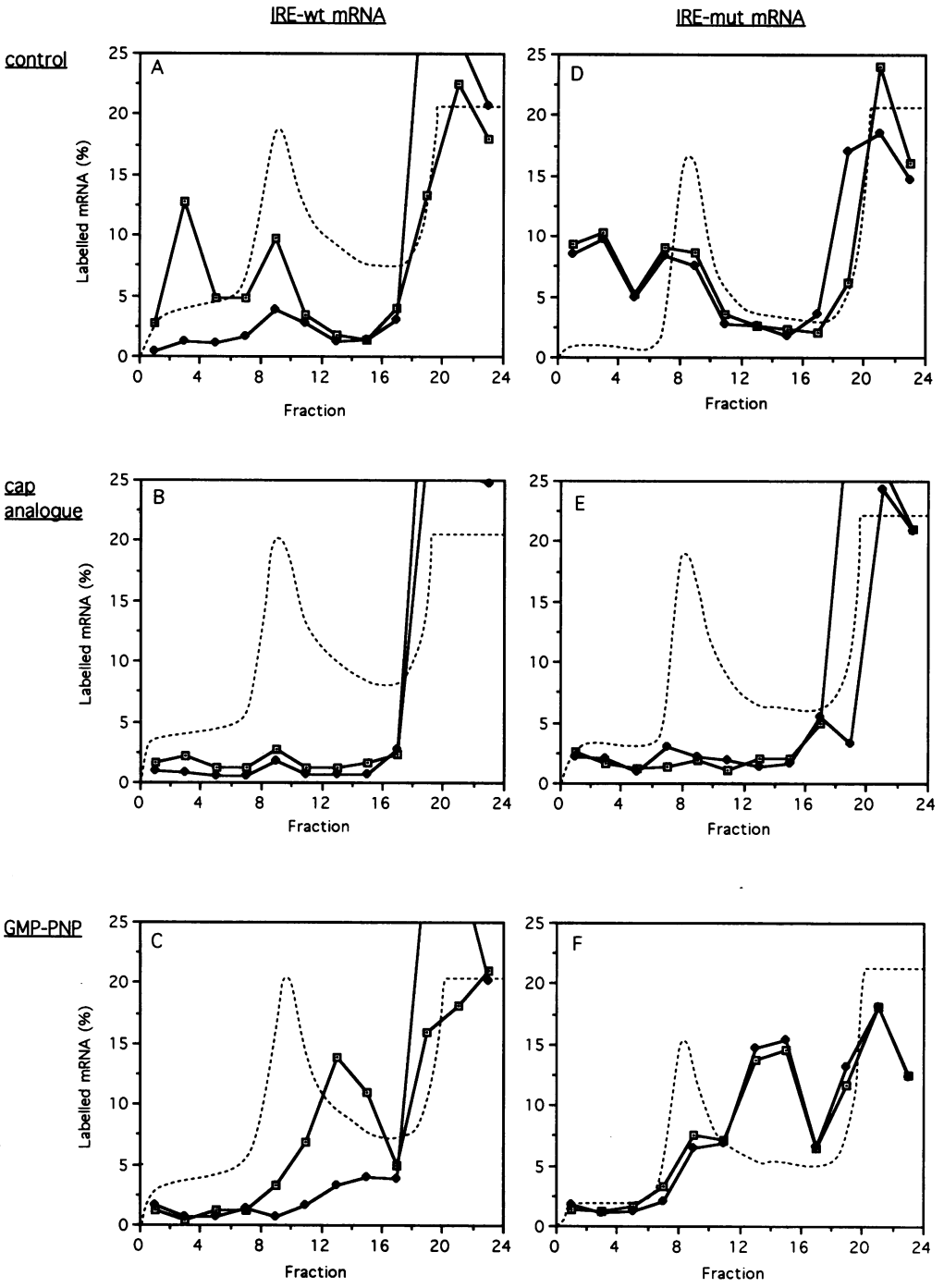

Fig. 5. Sucrose gradient profiles of translation initiation assays performed in rabbit reticulocyte lysate. Shortened mRNA transcripts of IRE-wt (**A**–**C**) and IRE-mut (**D**–**F**) were assayed in the presence of either 45 ng IRE competitor RNA (dotted squares) or 125 ng recombinant IRP (filled diamonds) and analysed on 5–25% linear sucrose gradients. The assays contained either no analogue (A and D), the m^7GpppG cap analogue (B and E) or GMP-PNP (C and F). The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A_{254} absorption profile, which was identical for gradients with added IRE or IRP.

obtained by sucrose gradient analyses of ferritin mRNA isolated from rats that had been injected with iron salts or the iron chelator desferrioxamine, in which repressed ferritin mRNA sedimented in the upper fractions of the gradient, are fully consistent with the proposed mechanism (Aziz and Munro, 1986).

It is important to consider the technical validity of our experimental approach, inasmuch as the results of the analysis involving full-length mRNAs suggested that the repressed mRNA co-sedimented with the small ribosomal subunit (Figure 2). This result appeared to indicate that the small ribosomal subunit (as part of the 43S pre-initiation complex) binds to the mRNA and that the IRE–IRP complex blocks its movement towards the initiator codon. We believe this not to be the case for the following reasons. The use of short transcripts and the translation initiation inhibitors m^7GpppG, GMP-PNP and edeine allow accurate identification of different pre-initi-

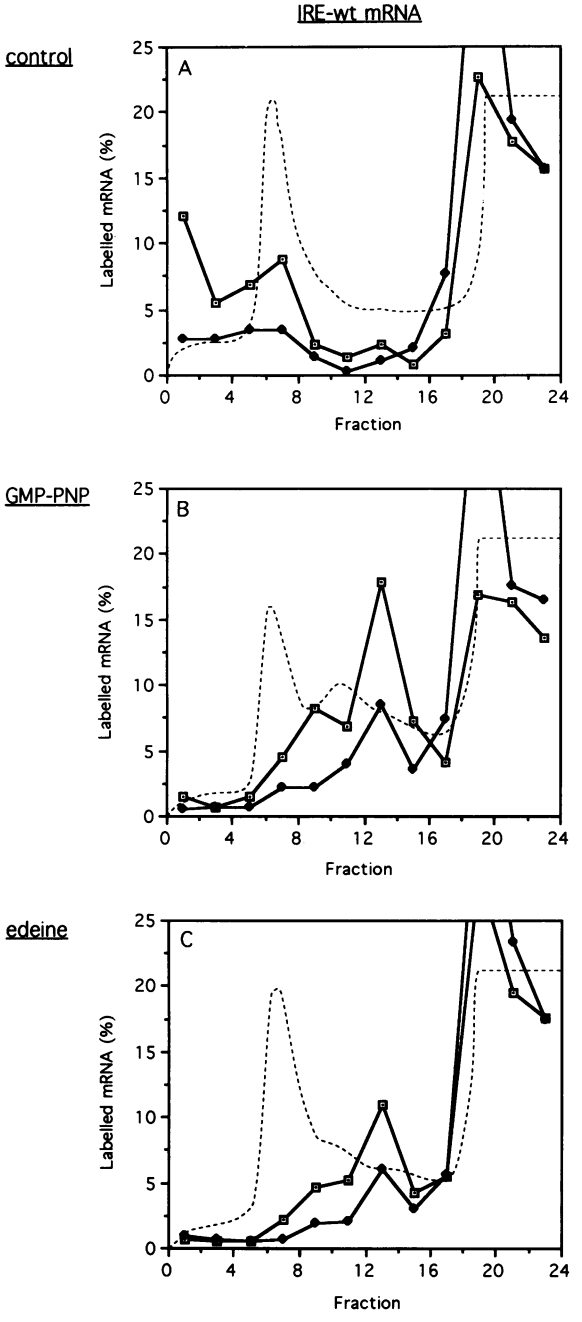

Fig. 6. 43S pre-initiation complexes not associated with the initiator codon remain bound to the mRNA during sucrose gradient analysis. Shortened IRE-wt mRNA was assayed in rabbit reticulocyte lysate in the presence of 45 ng IRE oligoribonucleotide (dotted squares) or 125 ng recombinant IRP (filled diamonds) and analysed on 5–25% linear sucrose gradients. The assays contained either no further additions (**A**), GMP-PNP (**B**) or edeine (**C**). The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A_{254} absorption profile, which was identical for gradients with added IRE or IRP.

ation complexes based on their sedimentation through sucrose gradients. The data obtained in translation initiation extracts from both a plant and a mammalian cell source show that IRP displaces mRNAs into fractions that co-sediment with mRNAs not bound by ribosomal subunits and that IRP prevents the accumulation of 43S pre-initiation complexes otherwise seen in the presence of GMP-PNP. Moreover, several lines of evidence suggest that a 43S pre-initiation complex, once associated with the mRNA, will remain bound to it throughout the analytical procedure. First, glutaraldehyde fixation of translation complexes prior to centrifugation through sucrose gradients was employed for the experiments using wheat germ extract, but was omitted from samples analysed in rabbit reticulocyte lysate. Both approaches yielded similar results. Second, a proportion of mRNAs sediment faster than 48S in the presence of GMP-PNP (Figures 5C, 6B and 8B). The presence of these complexes suggests that more than one 43S pre-initiation complex is associated with some of the mRNAs. Since the CAT ORF begins with the most 5′ AUG codon of the mRNA, this result implies that at least some 43S complexes remain bound to the mRNA independent of stabilizing interactions between the pre-initiation complex and an AUG codon. Third, the data obtained with edeine, which interferes with AUG recognition by the 43S complex, also support this interpretation. Fourth, hybridization of an antisense 2′-*O*-allyl oligoribonucleotide to a region of the 5′ UTR including the AUG codon itself permits the binding of the 43S pre-initiation complex, but inhibits its progression to the AUG codon. Under these conditions, it remains associated with the mRNA throughout the analytical procedure described here (H.E.Johansson and M.W. Hentze, manuscript in preparation).

Based on our results and the above considerations, we propose the model illustrated in Figure 8 for the regulation of ferritin and eALAS mRNAs by IRP, in which translational repression by IRP is a result of the IRE–IRP complex blocking access of the 43S pre-initiation complex to the mRNA. It remains to be determined whether IRP exerts its effect via direct steric hindrance of the 43S pre-initiation complex or whether it interferes with initiation factors which are thought to interact with the mRNA prior to the 43S complex and assist in its binding (the 'additional factors' in Figure 8). Elucidation of further molecular details concerning these points will require the development of suitable analytical techniques and will be the focus of further study.

General sensitivity of mRNAs towards steric interference with 43S association by RNA binding proteins

Having obtained information on the mechanism of ferritin regulation, it was of interest to examine whether steric repressors inhibit translation in a manner similar to IRP. The data obtained with the U1A protein show that it, too, prevents access of the 43S pre-initiation complex to U1A-wt mRNA. The data reveal that the cap-proximal region of eukaryotic mRNAs represents a sensitive target for translational repressor proteins to sterically block 43S pre-initiation complex binding to the mRNA (Figure 8) and suggest that this may be a more general mechanism for translational repression by RNA–protein complexes.

Multiplicity of mechanisms for translational control by mRNA binding proteins

Numerous examples of translational control by repressor proteins have been reported from a wide range of

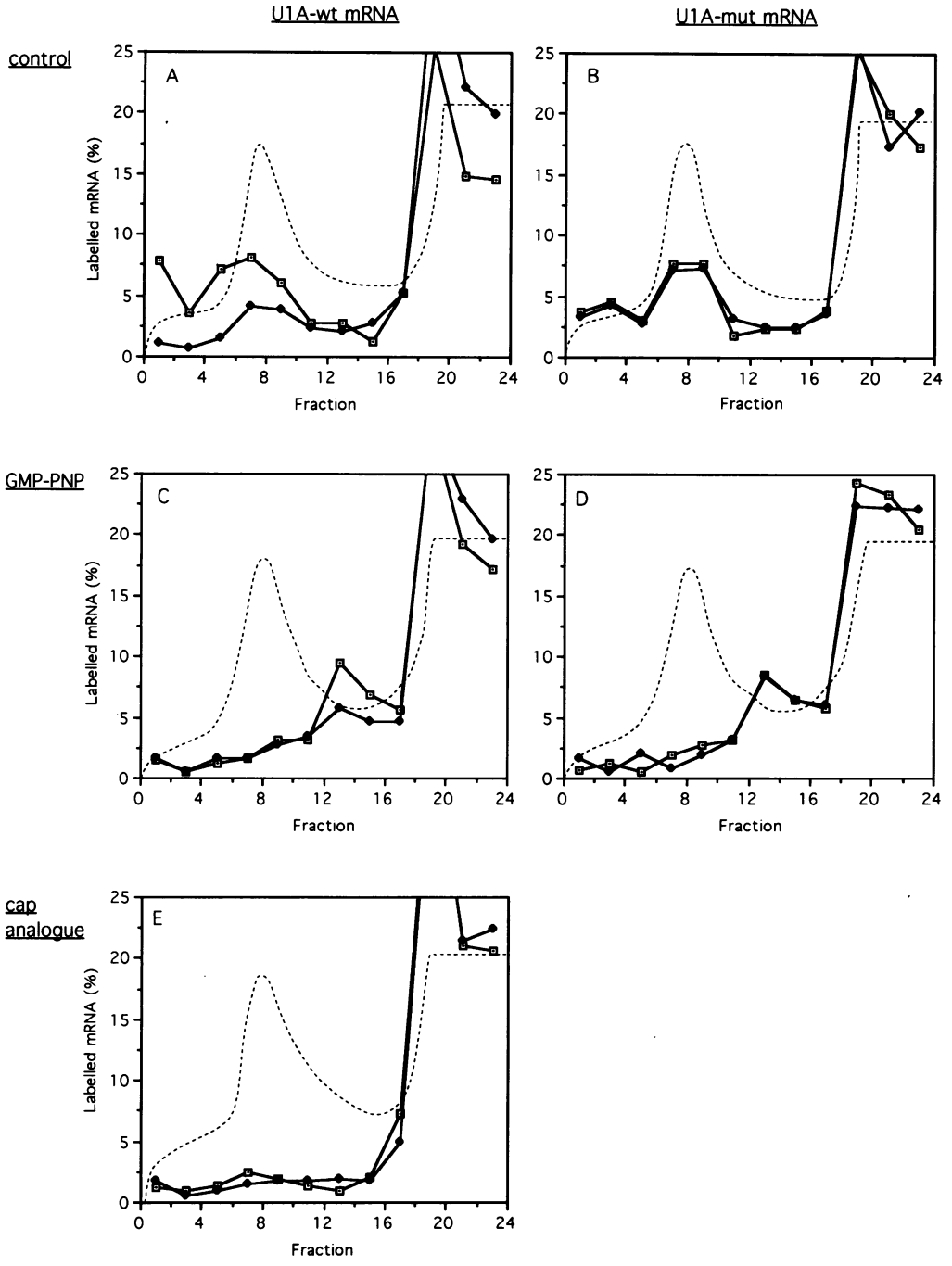

Fig. 7. U1A as a steric repressor of translation also prevents 43S complex association. Shortened transcripts of U1A-wt (**A**, **C** and **E**) and U1A-mut (**B** and **D**) were assayed in rabbit reticulocyte lysate in the absence (dotted squares) or presence (filled diamonds) of 700 ng recombinant U1A protein and analysed on 5–25% linear sucrose gradients. The assays contained either no analogue (A and B), GMP-PNP (C and D) or the m^7GpppG cap analogue (E). The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A_{254} absorption profile, which was identical for gradients −/+U1A protein.

eukaryotic organisms (summarized in Gray and Hentze, 1994). To our knowledge, this study on IRP-mediated regulation of ferritin and eALAS mRNA translation represents the first in which the interaction of a repressor protein-regulated mRNA with the eukaryotic translational apparatus has been characterized in detail. It is therefore a matter of speculation whether and which other mRNAs are controlled similarly to ferritin and eALAS. The lack of effect of IRP on translation when an IRE is located within the 3′ UTR of an mRNA (Casey *et al.*, 1988) appears to exclude translationally regulated mRNAs like 15-lipoxygenase mRNA, where the *cis*-acting regulatory sequences lie within this region (Ostareck-Lederer *et al.*, 1994). Likewise, the position dependence of IRP function strongly suggests that regulatory sequences located within the ORF or in a cap-distant position within the 5′ UTR, as in the thymidylate synthase mRNA (Chu *et al.*, 1993), contribute to translational regulation by different mechanisms.

Some examples of translational control, however, share

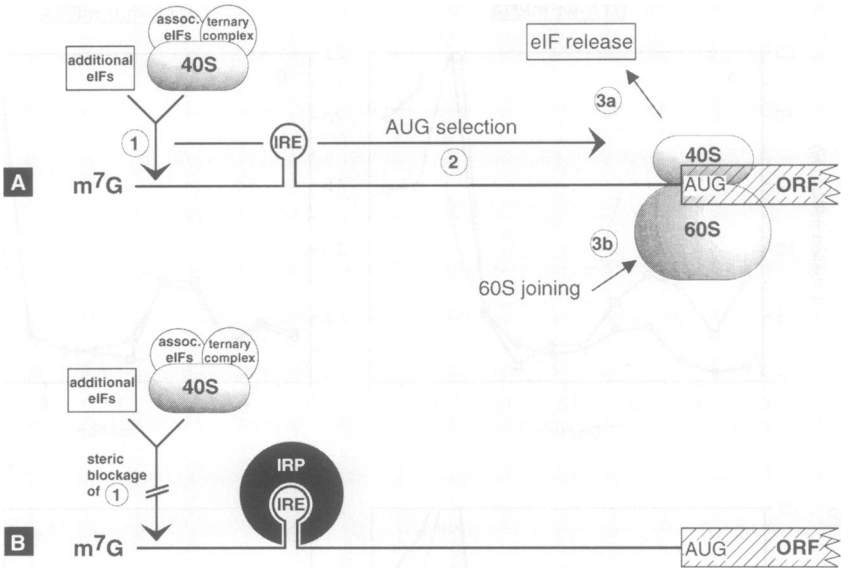

Fig. 8. Model of ferritin and eALAS regulation by IRP. (**A**) With IRP not bound to the IRE, (1) binding of the 43S pre-initiation complex to the mRNA is assisted by initiation factors associated with this complex, as well as additional eIFs which interact with the mRNA to facilitate 43S association. Subsequently (2), the 43S pre-initiation complex moves along the 5′ UTR towards the AUG initiator codon, (3) GTP is hydrolysed, initiation factors are released and assembly of the 80S ribosome occurs. (**B**) With IRP bound to the IRE, access of the 43S pre-initiation complex to the mRNA is (sterically) blocked. U1A bound to an mRNA in a position similar to that of the IRE also prevents step 1.

similarities with the IRE–IRP system. The regulation of a family of mRNAs involved in spermatogenesis in *Drosophila melanogaster*, including Mst87F, is mediated by a conserved element in the 5′ UTR of these mRNAs. As with ferritin, the position of this element with respect to the cap structure is conserved and movement of this element further downstream severely impedes regulation (Kempe *et al.*, 1993). In addition, the mRNAs encoding different ribosomal proteins are translationally regulated via cap-proximal elements in many species, ranging from L32 in *Saccharomyces cerevisiae* (Dabeva and Warner, 1993) to L30, L32 and S16 in human cells (Levy *et al.*, 1991). Conceivably, differences in the regulatory mechanisms may exist even within this family. A requirement for cap proximity has been demonstrated for the murine S16 mRNA (Hammond *et al.*, 1991). Unfortunately, the putative regulatory proteins that bind to these translationally regulated mRNAs have not yet been purified and demonstrated to function *in vitro*, leaving a final assessment of their functional kinship with IRP as a task for the future. It appears as if multiple mechanisms can operate within eukaryotic cells by which proteins regulate the association of an mRNA with the translational apparatus. In principle, the biochemical approach employed here should be applicable to many of these systems.

Materials and methods

Plasmid constructs and in vitro transcription of full-length and shortened mRNAs

The construction of the plasmids utilized for *in vitro* transcription by T7 RNA polymerase, IRE-wt (previously called I-12.CAT), IRE-mut (previously called I-19.CAT), UIA, NOP1, UIA-wt and UIA-mut, have been previously described (Stripecke and Hentze, 1992; Gray *et al.*, 1993). *In vitro* transcriptions optimized to yield >95% capping efficiency were performed as described (Gray *et al.*, 1993), excess cap analogue being removed after transcription by two rounds of G50 chromatography.

The α-^{32}P-labelled mRNAs were transcribed as above, with the exception that the final UTP concentration was reduced to 0.5 mM and 2.5 μM [α-^{32}P]UTP (800 Ci/mmol) (Amersham International, Amersham, UK) for full-length (8×10^6 c.p.m./μg) or 5 μM [α-^{32}P]UTP for shortened transcripts (1.6×10^7 c.p.m./μg) were included. IRE competitor oligoribonucleotides (34 nucleotides) were transcribed and purified as described (Gray *et al.*, 1993).

Cell-free translations

For translations in micrococcal nuclease-treated rabbit reticulocyte lysate (RRL) (Promega, WI), U1A (1 ng) and 2.5 ng full-length IRE-wt mRNA were translated in 12 μl reactions in the presence of 125 ng recombinant human IRP purified from *Escherichia coli* or 15 ng IRE competitor oligoribonucleotide (Gray *et al.*, 1993). Buffer N (150 mM KOAc, 24 mM HEPES, pH 7.6, 1.5 mM $MgCl_2$, 5% glycerol) was added to those translations which did not receive IRP. Translation proceeded at 30°C for 60 min and [^{35}S]Met-labelled translation products were subjected to SDS–PAGE and fluorography.

Translation initiation assays and sucrose gradient analysis

Wheat germ extract (WGE) was prepared as described (Gray *et al.*, 1993). Extracts competent for translation were incubated at 25°C (WGE) or 30°C (RRL) for 3 min with 0.5 mM cycloheximide (Sigma, Deisenhofen, Germany) prior to addition of mRNA. Where indicated, inhibitors of translation were incubated alongside cycloheximide at final concentrations of 1–2 mM GNP-PNP (Boerhinger Mannheim, Mannheim, Germany), 0.5 mM m^7GpppG cap analogue (New England Biolabs, MA) or 2 μM edeine (a gift from Dr R.Jackson, Cambridge, UK). Cycloheximide (0.1 mM), 0.5 mM GMP-PNP, 0.2 mM m^7GpppG and 1 μM edeine were shown to be sufficient to completely inhibit [^{35}S]Met incorporation (data not shown). Reactions in WGE (45 μl) were subsequently programmed with 1 ng UIA mRNA and 3 ng full-length or 0.75 ng shortened CAT mRNAs (see Figure 1). Reactions in RRL (48 μl) were programmed with 2 ng UIA or 2 ng NOP1 and 1.5 ng shortened CAT mRNAs. Forty five nanograms of IRE competitor transcripts (RRL), 125 ng recombinant human IRP or buffer N were added to mRNA prior to addition to the initiation extract. Recombinant human UIA protein (700 ng) purified from *E.coli* (Stripecke and Hentze, 1992) or its storage buffer was mixed with mRNA 15 min prior to addition to lysate. Initiation assays at 25°C (WGE) or 30°C (RRL) were stopped 5 min after the addition of mRNA by increasing the total volume to 100 μl with ice-cold dilution buffer (150 mM KOAc, 20 mM HEPES, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT) and 0.2% glutaraldehyde (WGE) and placed immediately on ice for 5 min. Translation initiation complexes

were resolved on 5 ml linear sucrose gradients (10–30% or 5–25%) containing 150 mM KOAc, 20 mM HEPES, pH 7.6, 5 mM $MgCl_2$ and 1 mM DTT. Gradients were centrifuged at 4°C in a Beckman SW50.1 rotor at 40 000 r.p.m. for 90 min (WGE) or at 30 000 r.p.m. for 180 min (RRL). Following centrifugation, 24 fractions of 210 µl were collected from the bottom of the gradient using a Pharmacia P-1 peristaltic pump and a Pharmacia RediFrac fraction collector. Absorption traces were recorded using a Pharmacia UV HR-10 flow cell with an A_{254} filter. Alternating fractions were counted by the Cerenkov method in a Beckman LS6000SC scintillation counter. RNA was extracted from 100 µl of the fractions, either directly (RRL) or after proteinase K treatment (WGE), and resolved on 4% denaturing polyacrylamide gels.

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